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Research Article

First global registration of an isolate producing aflatoxin B1 from the fungus (*Rhizopus microsporus*) from stored grains and nuts in Iraq

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Abstract
This study delineates the mycotoxin-producing capabilities of *Rhizopus microsporus* strains isolated from stored grains and nuts. Aflatoxins, particularly aflatoxin B1, are significant due to their deleterious effects on human and animal health and their economic impact on vital agricultural commodities. Employing polymerase chain reaction (PCR) for identification, one isolate was confirmed to produce aflatoxin B1 and was associated with *R. microsporus*. Thin-layer chromatography (TLC) and ammonia gas tests substantiated the isolate's toxin-producing ability. High-performance liquid chromatography (HPLC) quantified aflatoxin B1 concentration, revealing a yield of 395.19 PPb. This study's findings are unprecedented, marking the first known instance of such isolates producing toxins globally. DNA extraction from fungal hyphae, PCR analysis, nucleotide sequencing, and submission to the National Center for Biotechnology Information (NCBI) database further corroborated the isolate's classification as *R. microsporus*. The isolate was formally registered in the Gene Bank with serial number OP205376.1, representing a notable contribution to Iraqi and international scientific communities.

Keywords: Aflatoxin B1, HPLC (High-performance liquid chromatography), Mycotoxin, NCBI, *Rhizopus microspores*

INTRODUCTION

Fungi are a modern challenge due to their widespread distribution. Since they may grow in various human meals, including raw ingredients, contributing to bacterial food degradation (Gizachew *et al*., 2016). Some fungi in the food chain create poisons that can harm humans in developed and underdeveloped countries (Hymery *et al*., 2014). It is mainly responsible for visible and invisible changes, such as unpleasant odors and unpalatable flavors, which lead to food spoilage and significant economic losses .This causes visible and invisible changes, such as unpleasant scents and tastes, that cause food degradation and significant financial losses (Garnier *et al*., 2017). Some fungi can synthesize mycotoxins. The low molecular weight prevents the immune system from recognizing these things. Thus, they accumulate in the spleen, kidneys, and liver. They are among the most dangerous toxins due to their heat resistance(Okasha *et al*., 2024).

In addition to immune system suppression, these poisons cause congenital malformations, renal toxicity, and fetal teratogenesis. When ingested consistently in meals with varied concentrations, these toxins can cause chronic and acute central nervous system and blood vessel disturbances (Bhat and Vasanthi, 2003). Mycotoxins are secondary metabolic products produced by filamentous fungi and under appropriate temperatures and humidity. Poisoning with them can occur through ingestion, inhalation, or contact with the skin. Humans can consume them directly through food contaminated with toxins or by eating foods such as meat, milk, or eggs. Several fungi produce aflatoxins, which are carcinogenic, immunosuppressive, mutagenic, and poisonous. They contaminate maize, cotton, and peanuts. Aflatoxins reduce crop productivity and food safe-

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ty, endangering humans and animals (Guan *et al*., 2021). Filamentous fungi produce mycotoxins under ideal temperatures and humidity. These compounds can be poisoned by ingestion, inhalation, or skin contact. Consuming contaminated food or meat, milk, or eggs from animals fed contaminated feed can expose humans to toxins (Majeed *et al*., 2018).

Food toxins can cause cancer and damage the immune system. Twelve mycotoxins have been recognized as harmful to human health and common in food (Mustafa *et al*., 2021).Specific fungal species produce aflatoxins, which are naturally distributed worldwide. They can contaminate food, creating health and economic risks. They destroy 25% of worldwide crop productivity annually. Due to their abundance in most staple foods, aflatoxins B1, B2, G1, and G2 are particularly dangerous in many countries (Alameri *et al*., 2023)

The International Agency for Research on Cancer classifies AFB1 as the most common Group A liver and bile duct carcinogen. Toxin-producing fungi thrive in stored crops, causing fungal poisoning. Poor storage conditions, such as excessive humidity and variable temperatures cause fungal contamination. Poor harvesting practices also contribute. Drying, packaging, storage, and transportation increase fungal transfer and mycotoxin risk (Oadi, 2020).

Oadi (2020) reports that the production of 500 known mycotoxins has remained undiscovered, with 1,000 remaining undiscovered. Due to a lack of study and identification methods, fungal toxins are dangerous (Horky *et al*., 2018). *Rhizopus* is a Zygomycetes fungi found in soil, fruits, trees, decaying organic waste, and stored grains (Agrios, 1972). Zheng *et al*., 2007found it in warehouses, whereas Agrios (2005) related it to fruit and vegetable hard rot. *Rhizopus* species can damage harvested sweet potatoes and strawberries. This deterioration impacts crop look and flavor. Some species release plant poisons like rhizoxin, which can poison humans. The opportunistic pathogen *Rhizopus* can infect animals and humans, especially those with weaker immune systems (Ma *et al.,* 2009). The present study aimed to find out aflatoxin-producing isolate of type B1 from *Rhizopus microsporus* in Iraq, diagnosing it at the molecular level, registering it in the GenBank, and measuring its level of toxicity, that grows on grains, nuts, spices, dried fruits, and other food products.

MATERIALS AND METHODS

Collect and analyze samples

In February 2022, different nuts (walnuts, field pistachios, cashews, hazelnut, almonds) and grains (wheat, barley, maize) were collected from local markets in Karbala Governorate. Each type was randomly selected and 250 grams of each were taken. The samples were then stored in Polyethylene bags and transported to the Postgraduate Research Laboratory at the College of Applied Medical Sciences, University of Karbala.

Identification and detection of fungus

Each sample (nuts and grains) was individually subjected to surface sterilization using a 2% concentration of sodium hypochlorite for two minutes. Subsequently, the samples were washed with sterile distilled water and transferred onto container dishes placed on blotting paper to facilitate drying. The dried samples were then planted onto 9 cm diameter dishes containing sterile PDA medium. The planting process was carried out at a temperature of 121 ˚C and a pressure of 1 atmosphere for 15 minutes, utilizing a cooling kit. The plate was inoculated with four seeds placed at the outer edge, and an additional seed was placed at the center of the dish. The dishes were placed in an environment with a constant temperature of 29 ˚C for 7 days (Gupta and Kumar, 2020). Following the completion of the incubation period, the fungal isolates were refined by transferring a disc with a diameter of 5 mm from each colony and placing it in another dish containing the identical culture medium. The method was performed multiple times to obtain pure fungal isolates. The fungi were then identified based on their phenotypic traits using taxonomic keys developed by Pitt and Hocking (2009) and Raper and Fennell (1965).The appearance rate is calculated by dividing the number of samples in which the fungus appeared by the total number of samples and multiplying the result by 100.

Maintaining fungal isolates

The fungal specimens were stored in uncontaminated, aseptic glass tubes filled with Potato Dextrose Agar (PDA) media, positioned at an inclined angle. Specimens were placed on the culture medium in each tube and incubated at a temperature ranging from 25 to 37° C for one week. Subsequently, they were stored in the refrigerator until ready for use.

Evaluating the capacity of fungal isolates to generate aflatoxins

The ammonia test discovered isolates that produced aflatoxins. These isolates were cultured on coconut media that had been prepared beforehand. Pure colonies of the isolated fungus, which were seven days old and grown on PDA medium, were inoculated onto the coconut media. The cultures were then incubated at 29°C for ten days. Once the mycelium emerged, filter sheets were positioned and dampened with solution droplets. A 20% concentration of ammonia was put into the lids of the dishes where the fungi grew. The dishes were then inverted and incubated for four days. The toxin-producing fungi were differentiated from other fungi based on the red or orange coloration of the colony bases, as opposed to the translucent color (Saito

and Machida, 1999)

Aflatoxins were detected using thin-layer chromatography (TLC) technology. The fungal isolates were cultivated on PDA medium by inoculating 5 mm diameter discs of the examined fungus, one week old, in the middle of each dish. Each fungal isolate was replicated three times. The replicates were then incubated at a temperature ranging from 25 to 37°C for a week. After incubation, a dish from each isolate was selected and the culture medium on which the fungal isolate had grown was cut into small pieces using a sterile knife. These pieces were transferred to an electric blender containing 20 ml of chloroform using a sterile needle. The mixture was blended for 10 minutes. The mixture was subsequently filtered via filter paper. The resulting filtrate was transferred to a clean and sterile beaker. The quantity was reduced to around 1 ml in an electric oven set at a temperature of 40°C. The presence of aflatoxin B1 was detected using the TLC thin layer plate chromatography technique with dimensions of 20 × 20 cm, following the method described by Al-Jumaili (1996).

Before usage, the plates were heated in an electric oven at 120°C for one hour. The separation system employed for this purpose involved the employment of chloroform: methanol (95:5) mixture. A linear mark was made on the TLC plate, precisely 1.5 cm away from the bottom edge of the plate. A volume of 15 microliters of the standard poison AFB1 was extracted using a capillary tube and deposited on the line, precisely 2 cm away from the left border of the plate and 2 cm away from the area designated for the first standard poison. Subsequently, the samples were positioned. The fungal extract was obtained using the same procedure and in an equivalent quantity as the standard toxin, following the same way as the other isolates. The spots were subsequently dried and transferred to a separation basin. The basin contained a mixture of chloroform and methanol in a volume/volume ratio of (95:5). The progress of the solution was observed until it reached a distance of roughly 2 cm from the upper end.

The plates were removed and subjected to laboratory drying for 5 minutes. Subsequently, they were inspected under ultraviolet radiation with a wavelength of 360 nm. The identification of aflatoxin B1 was accomplished by comparing the migration coefficient Rf and the fluorescence color of the standard toxin, as described by Sobolev and Dorner (2002).

Molecular Identification

Refers to the process of identifying and characterizing organisms using molecular techniques. The fungi recovered in this investigation were subjected to molecular diagnostics using two methods:

Firstly, molecular diagnostics were conducted by utilizing the genetic marker known as Internal Transcribed Spacer (ITS).

Secondly, molecular diagnosis involves identifying the entire genetic material of the isolated fungus using advanced next-generation sequencing technologies.

DNA extraction and purification

The DNA extraction and purification from pure mushroom colonies was conducted using the DNeasy Plant Kits, a commercial product developed by the German company QIAGEN. The procedure involved the following steps:

a- Obtained 100-200 mg of an uncontaminated colony of fungi that was 10 days old and transferred it to a sterile 1.5 ml test tube (Eppendorf tube). Then, 400 microliters of AP1 buffer solution to the tube. The specimen was pulverized using a sterile plastic pestle (Micro pestle) and agitated many times using a device. Utilized a vibrator (specifically a Vortex) to pulverize the sample thoroughly. The objective of this stage was to eradicate the fungus cells.

b. The mixture was placed in a tube and kept in a water bath at a temperature of 65°C for 10 minutes. The tube was manually shaken 2-3 times throughout the incubation period. The purpose of this stage was to examine the fungal cells.

c. Inject 130 microliters of buffer solution P3 into the tube containing the combination, then thoroughly blend the contents using a shaker and let it sit for 5 minutes in a cold environment. This stage causes the detergents in the buffer solutions to separate, as well as the proteins and polysaccharides of the fungus.

d. The tube was centrifugated at a velocity of 14,000 revolutions per minute for 5 minutes. Subsequently, the resulting liquid above the sediment was moved to a purple QIA shredder (A unique biopolymer shredding system in a microcentrifuge spin-column format) Mini spin-column tube equipped with a distinctive filter. Additionally, it was subjected to centrifugation at the same velocity as before but for two minutes. This tube's filtration system effectively eliminates most sediments and fungal cell debris.

e. Transfer the liquid that has passed through the filter to a new, clean 2 ml test tube. Then, add 700 µl of AW1 buffer solution to the tube and mix the contents using a small pipette.

f. Next, 650 microliters of the mixture were carefully transferred using a small pipette to a white DNeasy Mini spin column tube equipped with a specialized filter designed to purify DNA. The tube underwent centrifugation at a velocity of 8000 revolutions per minute for one minute, after which the filtrate was discarded. The remaining portion of the mixture was transferred to the identical tube and subjected to centrifugation at the same speed and duration while the filtrate was likewise discarded.

g- A volume of 500 microliters of buffer solution AW2

was introduced into the same tube and subjected to centrifugation at a speed of 8000 revolutions per minute for a duration of one minute. The liquid that passed through the filter was disposed of. Subsequently, 500 microliters of buffer solution AW2 were introduced into the same tube and subjected to centrifugation. The filtrate was extracted after being subjected to a speed of 14,000 rpm for two minutes. The objective of this procedure was to cleanse the DNA that is trapped within the filter.

i- A volume of 500 microliters of buffer solution AW2 was introduced into the same tube and subjected to centrifugation at a speed of 8000 revolutions per minute for one minute. The liquid that passed through the filter was disposed off. Subsequently, an additional 500 microliters of buffer solution AW2 was introduced into the identical tube and subjected to centrifugation. The filtrate was extracted after being subjected to a speed of 14,000 rpm for two minutes. The objective of this procedure was to cleanse the DNA trapped within the filter.

The PCR replication program commenced with a denaturation step, lasting 5 minutes at a temperature of 95° C, followed by 35 cycles comprised three stages: initial disintegration for 40 seconds at a temperature of 95°C, subsequent annealing for 40 seconds at a temperature of 55°C, and finally extension for one minute at a temperature of 95°C. The temperature was 72 °C. Subsequently, the reaction enters its concluding stage, characterized by a last expansion lasting 5 minutes at a temperature of 72°C. The reaction product was transferred via electrophoresis on a 1.5% Agarose medium after adding 5 microliters of ethidium bromide dye. The reaction results were examined using an ultraviolet instrument.

The bioinformatics analysis was performed for nucleotide sequencing and conducted to determine the sequence of nitrogenous bases.

Following the PCR replication process, the resulting products were sent to Macrogen Company in South Korea to determine the sequence of the nitrogenous bases in each fungal sample. Additionally, the Basic Local Alignment Search Tool (BLAST) program, available on the NCBI (National Center for Biotechnology Information) website (https://www.ncbi.nlm.nih.gov/), was utilized to assess the similarity between the studied mushrooms and internationally recorded fungi.

Quantifying the quantity of aflatoxin using High-Performance Liquid Chromatography (HPLC) technique

The procedures contained in the study were followed (Beyene *et al*., 2019), as they were employed to detect and quantify AFB1. The procedure involved the following steps:

1- Mixed 25 ml of the fungal filtrate with 25 ml of methanol and 25 ml of chloroform. Allow the mixture to sit on the shaker for one hour.

2- The sample underwent filtration using Whatman No.4 filter paper. Subsequently, 25 ml of 90% methanol was introduced, and the mixture was then separated using a separating funnel.

3- Poured the liquid that passed through the filter into a separating funnel container. Then, 25 ml of hexane and 25 ml of methanol, which were 90% pure, were added. The lowermost stratum containing methanol was extracted and dehydrated using a water bath.

4-Chloroform: The sample was mixed with distilled water in a 1:1 ratio (25 ml each) and then separated using a separating funnel. The lower layer was filtered using filter paper containing 10 grams of anhydrous sodium sulfate. The filtrate was collected and subjected to evaporation using a water bath set at a temperature of 50°C.

5. The specimens were stored in the freezer until the level of AFB1 was determined using HPLC technology.

The experiment was carried out in the laboratories of the Ministry of Science and Technology's Department of Environment and Water. A high-performance liquid chromatography device was utilized, specifically the German-made SYKAMN model. The carrier phase used was a mixture of acetonitrile and distilled water in a ratio of 70:30. The separation column employed was a C18 - ODS column measuring 25cm in length and 4.6mm in diameter, which was used to separate mycotoxins. A fluorescence detector with an excitation wavelength of 365nm and emission wavelength of 445nm was employed to detect mycotoxins. The flow rate of the carrier phase was set at 0.7 ml/min.

RESULTS AND DISCUSSION

Identification and detection of fungus

A total of 54 isolates from the genus *Rhizopus spp.* were isolated. The incidence rate and frequency of various types of grains and nuts are presented in Table 2. The fungus exhibited a notable and significant frequency of occurrence (10-70%) in the analyzed samples, prompting the need for meticulous investigation to determine the identity of this species and its potential for toxin production.

Aflatoxin B1 production capacity of fungal isolates

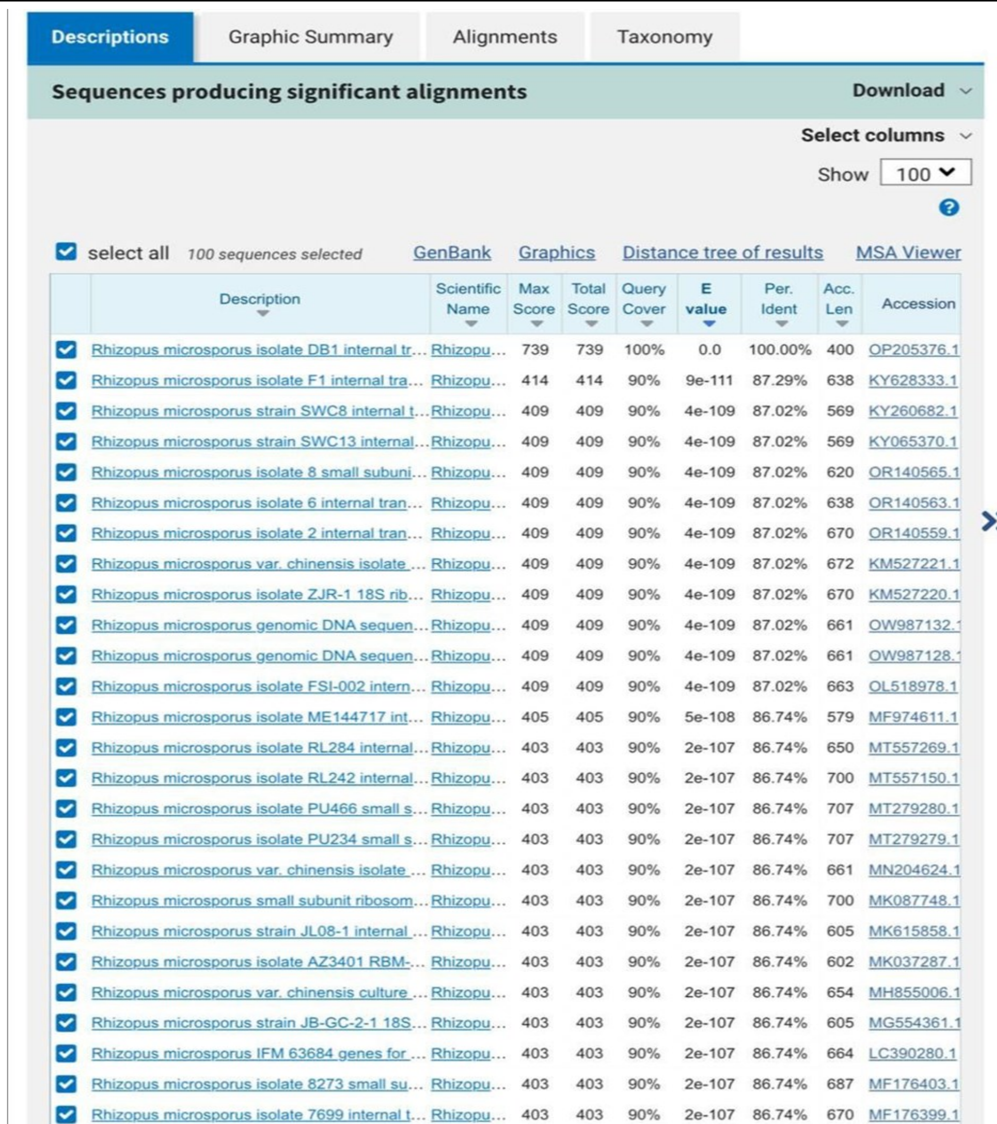
Ammonia methods: The detection of aflatoxin production by a fungal isolate obtained from nuts and grains was achieved using coconut and ammonia medium. This method involved observing a distinct color change in the bases of the colonies, with varying degrees of red coloration. The variation in color intensity may be attributed to the varying capacity of the isolates to generate aflatoxin, which aligns with the findings of Saito and Machida (1999), which is represented by the genus

Table 1. Primers used in the Polymerase chain reaction sequence technology

Primer		**Sequence** **5 ⟶ 3**	**PCR product size**
ITS1	F	TCC GTA GGT GAA CCT GCG G	500 – 800 bp
ITS4	R	TCC TCC GCT TAT TGA TAT GC	

Table 2. Showing the occurrence and frequency of the fungus *Rhizopus spp*.

Types of nuts and grains	**Frequency ratio %** **Rhizopus spp**	**Appearance rate %** **Rhizopus spp**
Maize	39.3	60
Wheat	12.2	20
Barley	10.9	30
Field pistachios	7.8	10
Almonds	25.3	20
Cashews	14.9	30
Walnut	25.4	20
Hazelnut	14.7	70



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download

Select columns

Show 100

select all 100 sequences selected GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Rhizopus microsporus isolate DB1 internal tr...	Rhizopu...	739	739	100%	0.0	100.00%	400	OP205376.1
Rhizopus microsporus isolate F1 internal tra...	Rhizopu...	414	414	90%	9e-111	87.29%	638	KY628333.1
Rhizopus microsporus strain SWC8 internal t...	Rhizopu...	409	409	90%	4e-109	87.02%	569	KY260682.1
Rhizopus microsporus strain SWC13 internal...	Rhizopu...	409	409	90%	4e-109	87.02%	569	KY065370.1
Rhizopus microsporus isolate 8 small subuni...	Rhizopu...	409	409	90%	4e-109	87.02%	620	OR140565.1
Rhizopus microsporus isolate 6 internal tran...	Rhizopu...	409	409	90%	4e-109	87.02%	638	OR140563.1
Rhizopus microsporus isolate 2 internal tran...	Rhizopu...	409	409	90%	4e-109	87.02%	670	OR140559.1
Rhizopus microsporus var. chinensis isolate ...	Rhizopu...	409	409	90%	4e-109	87.02%	672	KM527221.1
Rhizopus microsporus isolate ZJR-1 18S rib...	Rhizopu...	409	409	90%	4e-109	87.02%	670	KM527220.1
Rhizopus microsporus genomic DNA sequen...	Rhizopu...	409	409	90%	4e-109	87.02%	661	OW987132.1
Rhizopus microsporus genomic DNA sequen...	Rhizopu...	409	409	90%	4e-109	87.02%	661	OW987128.1
Rhizopus microsporus isolate FSI-002 intern...	Rhizopu...	409	409	90%	4e-109	87.02%	663	OL518978.1
Rhizopus microsporus isolate ME144717 int...	Rhizopu...	405	405	90%	5e-108	86.74%	579	MF974611.1
Rhizopus microsporus isolate RL284 internal...	Rhizopu...	403	403	90%	2e-107	86.74%	650	MT557269.1
Rhizopus microsporus isolate RL242 internal...	Rhizopu...	403	403	90%	2e-107	86.74%	700	MT557150.1
Rhizopus microsporus isolate PU466 small s...	Rhizopu...	403	403	90%	2e-107	86.74%	707	MT279280.1
Rhizopus microsporus isolate PU234 small s...	Rhizopu...	403	403	90%	2e-107	86.74%	707	MT279279.1
Rhizopus microsporus var. chinensis isolate ...	Rhizopu...	403	403	90%	2e-107	86.74%	661	MN204624.1
Rhizopus microsporus small subunit ribosom...	Rhizopu...	403	403	90%	2e-107	86.74%	700	MK087748.1
Rhizopus microsporus strain JL08-1 internal ...	Rhizopu...	403	403	90%	2e-107	86.74%	605	MK615858.1
Rhizopus microsporus isolate AZ3401 RBM-...	Rhizopu...	403	403	90%	2e-107	86.74%	602	MK037287.1
Rhizopus microsporus var. chinensis culture ...	Rhizopu...	403	403	90%	2e-107	86.74%	654	MH855006.1
Rhizopus microsporus strain JB-GC-2-1 18S...	Rhizopu...	403	403	90%	2e-107	86.74%	605	MG554361.1
Rhizopus microsporus IFM 63684 genes for ...	Rhizopu...	403	403	90%	2e-107	86.74%	664	LC390280.1
Rhizopus microsporus isolate 8273 small su...	Rhizopu...	403	403	90%	2e-107	86.74%	687	MF176403.1
Rhizopus microsporus isolate 7699 internal t...	Rhizopu...	403	403	90%	2e-107	86.74%	670	MF176399.1

Fig. 1. *Rhizopus microsprus DB1 isolate bearing the number OP205376 matches the global isolates in Gen Bank*

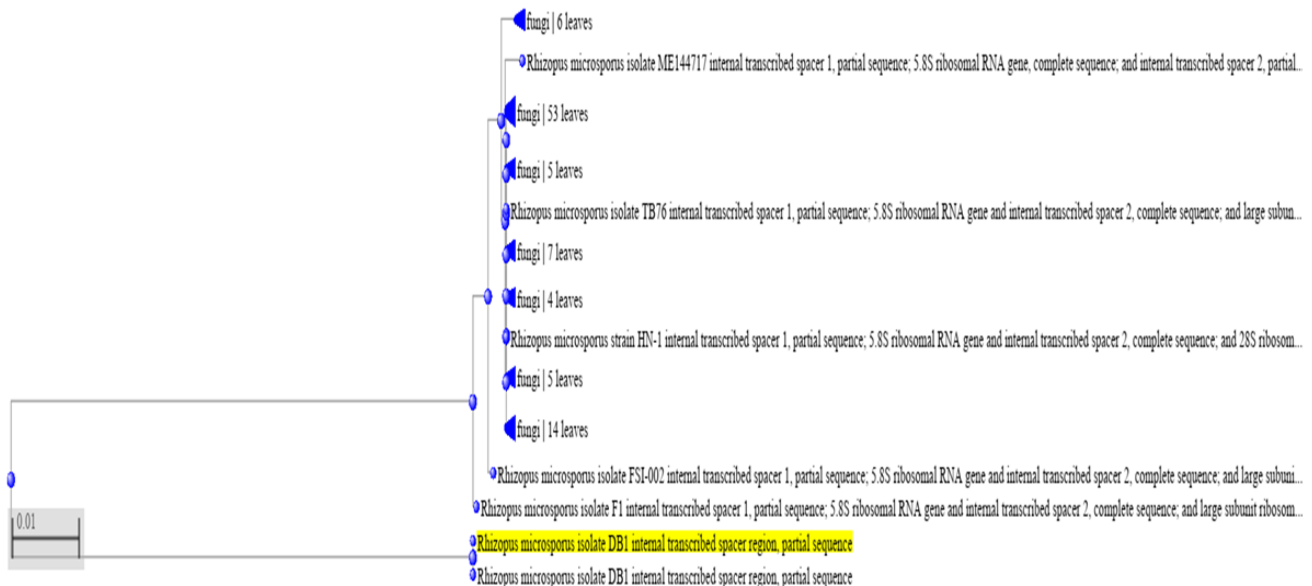


Fig. 2. *Genetic tree of the fungus OP205376 Rhizopus microsporus DB1 (marked in yellow).*

Aspergillus with a number of species (*A.flavus, A.parasiticus, A.oryzae, A.sojae*). They elucidated that the extent of red coloration is directly influenced by the amount of aflatoxin produced. A dark red isolate suggests its proficiency in generating larger quantities of isolates with light red or pink colony bases. The present study's findings demonstrate a specific isolate's capacity to generate aflatoxin.

Detection using Thin-layer chromatography (TLC) technique

Utilizing TLC technology, the chemical detection process demonstrated the capability of one of the isolates from the *Rhizopus spp*. genus. The isolate was chosen for molecular detection and genetic testing because of its strong fluorescence, which indicates the synthesis of aflatoxin B1.

Detection utilizing polymerase chain reaction (PCR) technique

The amplified gene products were submitted to Macrogen, a Korean company, for sequencing of the nitrogenous bases. The resulting sequences (Fig.3) were validated by comparing them with the existing information on this gene from the National Center for Biotechnology Information (NCBI) website, accessible at https://www.ncbi.nlm.nih.gov/nuccore. As per the BLAST Nucleotide program, the base sequences of the selected isolate were documented at NCBI by completing the registration form for the ITS18 gene (Fig. 1). This process resulted in the acquisition of a unique accession number for the local isolate. The phylogenetic tree (Fig.2) for the local isolate representing the genetic relationship was constructed following its alignment. The analysis of closely related strains in NCBI was conducted using the Blast Tree View tool. The isolate had a 100% match rate with international isolates, making it the first recorded instance of this isolate in the Gen Bank. It was assigned a unique identifier, as depicted in Fig.1. The region (Fig. 6) between ITS1 and ITS4 in the ribosomal gene S18 is regarded as stable and has been effectively utilized in differentiating various fungal species, providing essential diagnostic outcomes (Op De Beeck *et al*., 2014)

According to the registration information and the analysis of the proximity and similarity between the fungi, the results indicated that this species was the first to be registered in the World Gene Bank. To achieve a precise diagnosis, the genetic tree was utilized to ascertain the link between the species within each genus and the species under identification, together with their features and phenotypic traits. In traditional diagnosis, the classification of fungus relies on the crucial determination of the genotype. The SSU region is commonly employed in molecular categorization and diagnosis because it is easily amplified and exhibits significant variation, even among closely related species. The amplification of the SSU region of rRNA was employed with the specific objective of identifying the species. The DNA sequence was initially scrutinized to validate the nucleotide sequence and subsequently juxtaposed with worldwide strains. The NCBI-BLAST tool was employed to compare the results with worldwide strains, yielding precise outcomes. The Molecular Evolutionary Genetic Analysis (BLAST) program, a tool specifically developed for comparing sequences of similar genes, examining evolutionary links, and analyzing the pattern of DNA and protein evolution, was also employed (Aziz and Caetano-Anollés, 2021)

The global dissemination of this species can be attributed to its potential transmission via the import and

Fig. 3. *Showing the sequences of the nitrogenous bases of Rhizopus microsporus DB1*

12/10/2022 01:40 م — Chromatogram F:\ sample 1 .PRM — Page 1 of 1

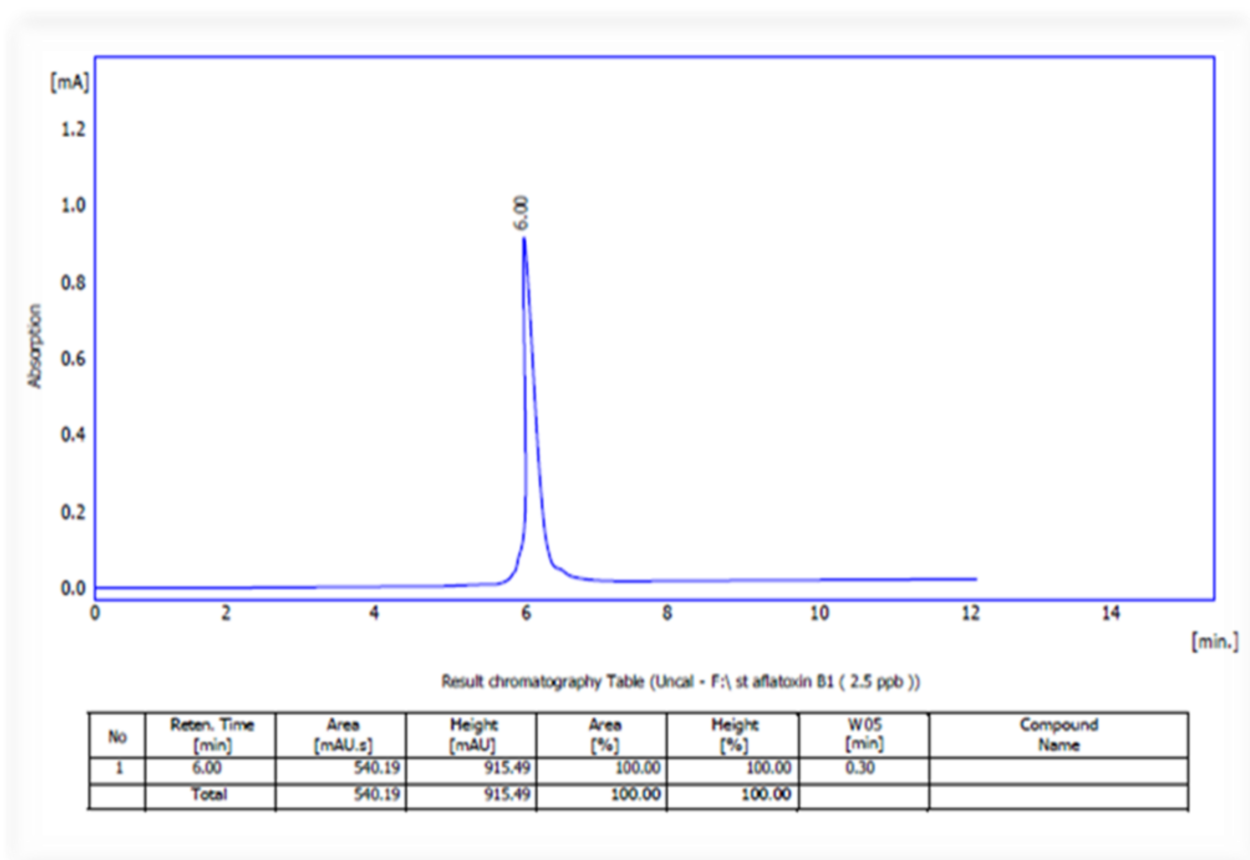
Chromatography Laboratory
HPLC

Sample Info:
Sample ID : sample 1
Sample : sample 1
Inj. Volume [mL] : 0.1
Amount 0
ISTD Amount 0
Dilution 1
Autostop : 20.00 min
Detector 1 : Detector 3
Subtraction Chromatogram : (None)
External Start : Start - Restart, Down
Range 1 : Bipolar, 2000 mAU, 10 Samp. per Sec.
Matching : No Change

Result chromatography Table (Uncal - F:\ sample 1))

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	3.80	9854.28	710.58	50.00	50.00	0.35	
2	6.00	652.19	180.59	20.00	20.00	0.10	
3	8.10	3652.55	610.59	30.00	30.00	0.20	
	Total			100.00	100.00		

Fig. 4. *Chromatogram of the concentration of aflatoxin B1 in the fungus R . microsporum DB1 by HPLC*



No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	6.00	540.19	915.49	100.00	100.00	0.30	
	Total	540.19	915.49	100.00	100.00		

Fig. 5. *Chromatogram of the standard for aflatoxin B1 at a concentration of 2.5PPb by HPLC*

export of diverse foodstuffs and items and through human carriers. Mycotoxin generation, especially aflatoxin, is associated with several environmental, chemical, and biological factors. Environmental factors encompass variables such as temperature and activity levels. Factors such as water quality, pH levels, and the length of incubation period and chemical aspects encompass the base material composition, nutrients availability, and presence of antifungal compounds. Biological factors involve the specific strain, the vaccination dosage administered, and other bacteria in competition. If these circumstances are conducive to the proliferation of toxin-producing fungus, the likelihood of poison production is definite, and vice versa. Toxins are produced when the fungus infects and colonises the food item (Kumar *et al*., 2021).

Most biological agents are used in the biological control program function by inhibiting the growth of mycotoxin-producing fungi within food items, typically without causing their death. Consequently, fungal growth becomes apparent when planting a food item infected with the toxic fungus (such as grains or manufactured foodstuffs) on a culture medium like PDA. This indicates that the fungus infects the food item. However, when testing for the toxin in the infected food item, the result was negative, indicating that the material was not contaminated with mycotoxin. This indicates that the fungus is found within the food item but cannot establish itself and spread throughout it (invasion of components). A significant proportion of the food items is confined to a narrow and restricted location(Savita and Sharma, 2019).

Quantification of toxin B1 in *Rhizopus microsporus* DB1

The HPLC instrument successfully detected the presence and concentration of aflatoxin B1. The results revealed that this technique is highly effective in identifying toxin production. The fungus demonstrated a toxin production rate of 395.19 ppb, as depicted in Fig.4. Upon comparing the observed peaks with the reference toxin diagram illustrated in Fig.5. It is evident that the peaks correspond, suggesting that the fungus was capable of producing aflatoxin B1. The study's findings revealed that this species was documented for the first time as a fungus capable of producing aflatoxin B1 in the grain stores of Kerbala governorate. Recording variation in the production of aflatoxin on pistachio occurred in different regions, as 13 toxin-producing isolates out of 120 isolates, all of which belong to the fungus *Aspergillus*, from 10 different Iranian cities, and included types B1, B2, G1, and G2 (Moghadam *et al*., 2020). The environmental conditions surrounding the fungi are considered one of the main causes of the fun-

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National Center for Biotechnology Information

Log in

Nucleotide | Nucleotide | Search | Advanced | Help

GenBank | Send to:

Change region shown

Customize view

Rhizopus microsporus isolate DB1 internal transcribed spacer region, partial sequence

GenBank: OP205376.1

FASTA Graphics

Go to:

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LOCUS       OP205376                 400 bp    DNA     linear   PLN 16-AUG-2022
DEFINITION  Rhizopus microsporus isolate DB1 internal transcribed spacer
            region, partial sequence.
ACCESSION   OP205376
VERSION     OP205376.1
KEYWORDS    .
SOURCE      Rhizopus microsporus
  ORGANISM  Rhizopus microsporus
            Eukaryota; Fungi; Fungi incertae sedis; Mucoromycota;
            Mucoromycotina; Mucoromycetes; Mucorales; Mucorineae;
            Rhizopodaceae; Rhizopus.
REFERENCE   1  (bases 1 to 400)
  AUTHORS   Alasady,D.F. and Alzobiady,B.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-AUG-2022) Applied Medical Sciences, Kerbala
            University, Mitham Al Tamar, Kerbala, 00964 00964, Iraq
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..400
                     /organism="Rhizopus microsporus"
                     /mol_type="genomic DNA"
                     /isolate="DB1"
                     /db_xref="taxon:58291"
     misc_RNA        <1..>400
                     /note="internal transcribed spacer region"
ORIGIN
        1 gagactcagg acatataagc tataatgggt aggcctgctg tgggatttga tcgatgccga
       61 tcaggatgac ctttcttcct ttgggaagga aggcgcctgg cacccttac catataccat
      121 gaattcagaa ttgaaagtgt aataaaatga gaactttgaa caatggagct cttggttctc
      181 gcattgatga aaaacgtcgc acagtgcgat acctagtgtg aattccatat tcgcgaattt
      241 gctagtcttt gaacgcagct tgcacccaag ggatcctctc tcacgtccgg ttgctagagt
      301 atcatagcca acccacacac aagatctatt ccatggggga atggacccat tcggtcagat
      361 ttgaagaccc gaccgactgt ctcagataca gcatacatgt
//
```

Analyze this sequence
Run BLAST
Pick Primers
Highlight Sequence Features
Find in this Sequence

Related information
Taxonomy

Recent activity
Turn Off Clear
Rhizopus microsporus isolate DB1 internal transcribed spacer Nucleotide
See more...

Fig. 6. *Rhizopus microsporus isolate DB1 internal transcribed spacer region, partial sequence*

gus producing toxins, including high temperatures and humidity, pH,and the food material on which the fungi grow(Thakur and Teja, 2024).

The emergence of this species can be attributed to mutations or environmental conditions that led to changes at the species level. Environmental conditions play a significant role in driving mutations and adaptations, resulting in the emergence of isolates with distinct characteristics from their ancestors. These changes enable the species to survive and thrive in its existing environment(Wani *et al*., 2022)

Conclusion

This fungus species *R. microsporus* isolate DB1no. OP205376.1, which produces aflatoxin B1 has garnered attention because of its detrimental impact on health. It can infiltrate the food chain and ultimately affect humans, leading to the development of numerous diseases and health issues. The endofungal bacteria present within *Rhizopus* species, isolated from foodstuffs, hold the potential to modulate the toxicity levels of the fungal host. This is mainly attributed to its re-

lease of aflatoxin toxins. Furthermore, this pathogen can be transferred by industrial means due to its contamination of grains often utilized in food industries, including corn oil and other products. This is evidenced by its ability to disseminate and infect the grains. Due to its production of aflatoxin toxins, this fungus poses a health hazard to humans and animals when they enter the consumer's body.

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Conflict of interest

The authors declare that they have no conflict of interest.

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